Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Testing of *Salmonella choleraesuis* Bacterins

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Supplemental Assay Method for Potency Testing *Salmonella choleraesuis* Bacterins

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing *Salmonella choleraesuis*, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.122. Mice are vaccinated twice, 14 days apart, and challenged with a standard dose of virulent *S. choleraesuis* 7-10 days after the second vaccination.

1.2 Keywords

Salmonella choleraesuis, potency, mouse, vaccination-challenge, 9 CFR 113.122, bacterin

2. Materials

2.1 Equipment/instrumentation

2.1.1 Bausch and Lomb Spec 20 spectrophotometer, or equivalent

2.1.2 Bunsen burner

2.1.3 37°C incubator

2.1.4 Micropipettors, 20-200 µl and 200-1000 µl

2.1.5 Vortexer

2.1.6 Crimper for aluminum seals on serum vials

2.1.7 Rotary shaker

2.2 Reagents/supplies

2.2.1 *S. choleraesuis* challenge culture, IRP SCC serial 5. This culture is available from the Center for Veterinary Biologics-Laboratory (CVB-L), United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Ames, IA.

2.2.2 Test bacterin(s) containing *S. choleraesuis*

2.2.3 APHIS-approved *S. choleraesuis* reference bacterin, IRP SCB serial 4. This reference bacterin expires on January 1, 2001. It is available from the CVB-L, USDA-APHIS, Ames, IA.

2.2.4 Syringes, 1 ml

2.2.5 Needles, 26 ga, 3/8 in

2.2.6 Glass serum bottle, 20-100 ml

2.2.7 Rubber stopper, 13 x 20 mm, and aluminum cap for serum bottle

2.2.8 Glass tubes, screw cap, 13 x 100 mm

2.2.9 Pipettes, 5 ml, 25 ml

2.2.10 Micropipette tips, up to 1000 µl capacity

2.2.11 Tryptose agar plates (for plate count)

2.2.12 Tryptose broth

2.2.13 Phosphate-buffered saline (PBS)

2.2.14 Brain-heart infusion broth

2.3 Animals

2.3.1 Mice, 16-22 g. Although the 9 CFR does not specify a specific mouse type or source, some colonies of mice may be relatively resistant to salmonellosis and therefore less suitable for this assay.

2.3.2 Sixty mice are required for each serial to be tested (20 mice/dilution x 3 dilutions/serial). Sixty additional mice are required for the reference bacterin. Thirty mice are required to determine the LD_{50} of the challenge inoculum. All mice must be from the same source colony and of similar weight and/or age.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in sterile technique, the handling of live bacterial cultures, and the handling of mice.

3.2 Selection and handling of test mice

3.2.1 Mice of either sex may be used, but females are recommended.

3.2.2 All mice must be housed and fed in a similar manner.

3.2.3 Identify each cage of mice by treatment group.

3.2.4 If any mice die after vaccination but prior to challenge with live *S. choleraesuis*, necropsy these mice to determine cause of death, if the cause of death is not outwardly apparent. If the cause of death is unrelated to vaccination, file the necropsy report with the test records, and no additional action is needed. If death is attributable to the test bacterin, report the death immediately to the Center for Veterinary Biologics-Inspection and Compliance (CVB-IC), which may request further safety testing of the bacterin.

3.2.5 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the mice and to sanitize contaminated rooms.

3.3 Preparation of supplies/equipment

3.3.1 Sterilize all glassware before use.

3.3.2 Use only sterile bacteriological supplies (pipettes, syringes, needles, rubber stoppers, diluents, etc.).

3.3.3 All equipment must be operated according to manufacturers' instructions and maintained and calibrated, as applicable, according to current CVB-L Standard Operating Procedures (SOPs).

3.4 Preparation of reagents

3.4.1 *S. choleraesuis* Bacterin. Reference bacterin IRP SCB serial 4. Expiration date January 1, 2001. Dilute this reference bacterin 1:5 in PBS immediately prior to use. (For purposes of calculating PD_{50}, the 1:5 dilution is considered undiluted when comparisons are made with the test serial.)

3.4.2 *S. choleraesuis* challenge culture. The challenge culture, IRP SCC serial 5, is lyophilized in 2-ml amounts. Store vials of lyophilized culture at 4°C or colder.

3.4.3 Phosphate-buffered saline (NVSL media #10559)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Sodium phosphate, dibasic	1.15 g
Potassium phosphate, monobasic	0.2 g
Reagent grade water	q.s. 1000 ml

Adjust pH to 7.2 ± 0.1. Autoclave 20 min at 121° C. Store at 20°-25°C for no longer than 6 mo.

3.4.4 Tryptose broth (National Veterinary Services Laboratories [NVSL] media #10404)

Tryptose broth powder (BBL or equivalent)	26 g
Reagent quality H_2O	q.s. 1000 ml

Autoclave 20 min at 121°C. Cool before using. Store at 20°-25°C no more than 6 mo.

3.4.5 Tryptose agar (NVSL media #10093)

Tryptose agar powder (BBL or equivalent)	41 g
Reagent quality H_2O	q.s. 1000 ml

Autoclave 25 min at 121°C. Cool in 56°C water bath.

Pour into sterile petri dishes. Allow to cool to room temperature. Store at 2°-7°C for no more than 6 mo.

3.4.6 Brain-heart infusion broth (NVSL media #10009)

Brain-heart infusion (BBL or equivalent)	37 g
Reagent grade water	q.s. 1000 ml

Autoclave 20 min. at 121° C. Store at 20°-25°C for no longer than 6 mo.

4. Performance of the test

4.1 Vaccination of test animals

4.1.1 Check the label on each product to confirm identity and dose volume.

4.1.2 Test each test bacterin and the reference bacterin at 3 fivefold dilutions. Typically, test the bacterins at 1:5 (e.g., 1 ml + 4 ml), 1:25, and 1:125 dilutions. **Reminder: An initial 1:5 dilution of IRP SCB serial 4 is made to create "undiluted" reference bacterin for purposes of this assay.** It is permissible to make fivefold dilutions other than those described as long as the reference and test bacterins are tested at the same dilutions. For viscous bacterins, it is advisable to start at 1:2 or 1:3 and make fivefold dilutions from this starting point to increase injectability of the product at the low dilution.

4.1.3 Thoroughly mix product by inverting end-to-end at least 10 times. Make the appropriate fivefold dilutions of the reference bacterin in PBS. Make identical fivefold dilutions of the test bacterin(s) in PBS or the diluent approved in the specific outline of production for that product. (Some oil-adjuvanted products require oil-based diluents.) Place each dilution in a separate sterile injection vial. Prepare dilutions immediately prior to use; do not store in diluted form.

4.1.4 Weigh 5 randomly selected mice immediately prior to vaccination to assure that the average body weight of the mice is between 16 and 22 g. Record weights.

4.1.5 Vaccinate separate groups of 20 mice with each of the 3 test bacterin dilutions and 3 reference bacterin dilutions. For reference bacterin groups, inject each mouse with 0.25 ml intraperitoneally. Inject test bacterins intraperitoneally at a dose volume that corresponds to 1/20 of the least dose recommended on the product label. This volume must not be <0.1 ml.

Note: It is permissible to vaccinate a few extra mice in each group to compensate for any potential deaths that may occur prior to challenge. However, if extra mice are vaccinated, all surviving at the time of challenge must be challenged with live *S. choleraesuis* and included in data calculations.

4.1.6 Revaccinate the mice in a similar manner 14 days after the first vaccination.

4.1.7 Retain 30 nonvaccinated mice to determine LD_{50} of the challenge.

4.2 Preparation of challenge

4.2.1 Reconstitute a vial of challenge culture in 1 ml brain-heart infusion broth.

4.2.2 Inoculate 3 tubes containing 10 ml of brain-heart infusion broth with 100 µl of reconstituted culture.

4.2.3 Incubate the inoculated tubes at 37° ± 1°C for 16-20 hr.

4.2.4 Perform a gram stain on the overnight culture, using the method described in the current version of BBSOP0004. If the bacteria in the gram stain are short, gram-negative rods (evidence of pure culture), proceed to the next step. If the challenge appears contaminated, discard affected tubes.

4.2.5 Inoculate 200 ml tryptose broth with 4 ml of the overnight culture. Incubate at 37° ± 1°C on a shaker (100 rpm) until the density is 25 ± 2% T at 620 nm using a Spec 20 spectrophotometer, approximately 4 hr.

1. Place the culture in a 13 x 100-mm screw-cap tube for spectrophotometric determination.

2. Use sterile tryptose broth in a 13 x 100-mm tube as a blank for the spectrophotometer.

4.2.6 Perform a gram stain on the standardized culture, as described in **Section 4.2.4**.

4.2.7 Prepare a 10^{-6} dilution of the standardized culture in tryptose broth. **This preparation, which is used to challenge the mice, will hereupon be called challenge inoculum.** Place challenge inoculum in a serum vial and seal with a rubber stopper and aluminum ring. Place in a serum vial and seal with a rubber stopper and aluminum ring. Save aliquot(s) of this inoculum in a separate vial; retain vial(s) as sample(s) for postchallenge plate counts.

4.2.8 Make 3 additional tenfold dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of the challenge inoculum to determine the LD_{50} of the challenge. Place each dilution in a separate serum vial and seal.

4.2.9 Place all vials of challenge on ice to transport to animal room. Keep on ice through challenge procedure and until challenge is added to plates for postinoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge all vaccinates 7-10 days after the second vaccination.

4.3.2 Challenge nonvaccinated LD_{50} controls at the same time as the vaccinates.

4.3.3 Inoculate each vaccinated mouse with 0.25 ml of challenge inoculum intraperitoneally, using a 26-ga, 3/8-in needle.

4.3.4 Inoculate separate groups of 10 nonvaccinated control mice intraperitoneally with 0.25 ml of each of the LD_{50} dilutions.

4.4 Postinoculation plate count

4.4.1 After mice are challenged, perform a plate count on the challenge inoculum according to the current version of BBSOP0019, using the vials retained for this purpose.

4.4.1.1 Use tryptose broth as a diluent, and plate on tryptose agar or 5% bovine blood agar. Incubate plates aerobically at 37° ± 1°C for 18-30 hr.

4.4.1.2 Calculate the colony-forming units (CFU) per challenge dose according to the following formula:

Average count in 0.1 ml x 2.5 x dilution factor (see table below)=CFU/0.25-ml dose of challenge culture

If plates used for avg. count were inoculated with:	Dilution factor
10^{-1} dilution of challenge inoculum	10
10^{-2} dilution of challenge inoculum	100
10^{-3} dilution of challenge inoculum	1000

4.5 Observation of mice after challenge

4.5.1 Observe the mice daily for 14 days after challenge. Record deaths.

4.5.2 If deaths occurring after challenge are suspected to be due to causes other than salmonellosis, necropsy such mice to determine the cause of death. If cause of death is unrelated to vaccination and/or challenge, do not include data from these mice in the total deaths for the test.

5. Interpretation of the test results

5.1 Interpret the test as prescribed in 9 CFR, Part 113.122.

5.1.1 Calculate the LD_{50} (theoretical dose/dilution at which the challenge would be lethal to 50% of the control mice) of the challenge inoculum using the Reed-Muensch method of estimation, or equivalent. A valid test must have an LD_{50} between 10 and 1,000.

5.1.2 Calculate the PD_{50} of the reference bacterin and each test bacterin (theoretical dose/dilution at which the bacterin would protect 50% of the mice) using the Reed-Muensch method of estimation, or equivalent.

5.1.2.1 At least 2 dilutions of the reference must protect >0% and <100% of the mice for a valid test.

5.1.2.2 If the PD_{50} of the reference cannot be calculated because the lowest dilution tested protects <50% of the mice, the serial may be retested, ***provided that*** the following conditions are met:

1. If the serial is not retested, it is unsatisfactory.

2. If the protection provided by the lowest dilution of the standard exceeds that provided by the lowest dilution of the test serial by at least 6 mice, the test serial is unsatisfactory without additional testing.

3. If the total number of mice protected by the reference (sum of survivors in all dilution groups) exceeds the total number

protected by the test serial by 8 mice or more, the test serial is unsatisfactory without additional testing.

5.1.2.3 If the PD_{50} of the test serial in a valid test cannot be calculated because the highest dilution protected more than 50% of the mice, the serial is satisfactory without further testing.

5.1.3 Divide the PD_{50} of each test serial by the PD_{50} of the reference to calculate the relative potency (RP) for each serial.

5.1.4 If the RP of the test serial(s) is $\geq$0.5, the serial is satisfactory.

5.1.5 If the RP of the test serial(s) is <0.5, the serial is unsatisfactory.

5.1.5.1 A test serial with an RP <0.5 may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. Calculate the results of the retests in the following manner:

1. Average the RP values of the retests.

2. If the average RP of the retests is less than 0.5, the serial is unsatisfactory.

3. If the average RP of the retests is >0.5 **AND** the RP obtained in the original test is 1/3 or less than the average (RP) of the retests, the test bacterin is satisfactory. Consider the initial test to be the result of test system error.

4. If the average of the retests is >0.5 **BUT** the RP of the original test is greater than 1/3 of the average RP of the retests, calculate a new average RP using the RP values obtained in all tests (original plus retests). If the new average RP is >0.5, the test bacterin is satisfactory. If the new average RP is <0.5, the test bacterin is unsatisfactory.

5.1.6 Record the plate count (CFU/dose) of the challenge on the test result form. This information is for informational purposes to track trends and to troubleshoot problem tests. The 9 CFR does not specify a minimum or maximum CFU/dose for this test.

6. Report of test results

Report the results of the test(s) as described by the current version of BBSOP0020.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.122, U.S. Government Printing Office, Washington, DC, 1999.

7.2 Reed LJ, Muench H, 1938. A simple method of estimating 50% endpoints. *Am J Hygiene*, 27:493-497.

8. Summary of revisions

This is a new SAM.